Effect of ADAMTS-13 on cerebrovascular microthrombosis and neuronal injury after experimental subarachnoid hemorrhage

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Abstract: Background: Microthrombosis and reactive inflammation contribute to neuronal injury after sub- arachnoid hemorrhage (SAH). ADAMTS-13 cleaves von Willebrand factor multimers, and inhibits thrombus formation and, seemingly, inflammatory reactions. Objective: To investigate the effect of ADAMTS-13 in experi- mental SAH. Methods: A total of 100 male C57/BL6 mice were randomly assigned to four groups: sham (n = 15), SAH (n = 27), vehicle (n = 25), and ADAMTS-13 (n = 23; 100 lL per 10 g of body weight of 100 lg of ADAMTS-13 per 1 mL of 0.9% NaCl; 20 min after SAH). Neurologic performance was assessed on days 1 and 2 after SAH. Animals were killed on day 2. The amounts of subarachnoid blood, microthrombi, apoptosis and degenerative neurons were compared. The degree of neuronal inflammation and vasospasm was also com- pared. In five mice each (SAH and ADAMTS-13 groups), bleeding time was assessed 2 h after SAH. Results: Systemic administration of ADAMTS-13 achieved significant amelioration of microthrombosis and improvement in neurologic performance. ADAMTS-13 reduced the amount of apoptotic and degenerative neurons. A ten- dency for decreased neuronal inflammation was observed. ADAMTS-13 did not show any significant effect on vaso- spasm. The degree of systemic inflammation was not changed by ADAMTS-13 administration. ADAMTS-13 neither increased the amount of subarachnoid blood nor prolonged the bleeding time. Conclusions: ADAMTS-13 may reduce neuronal injury after SAH by reducing micro- thrombosis formation and neuronal inflammation, thereby providing a new option for mitigating the severity of neuronal injury after SAH.

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Effect of ADAMTS13 on Cerebrovascular Microthrombosis and Neuronal Injury after Experimental Subarachnoid Hemorrhage

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Summary

Background: Occurrence of microthrombosis and reactive inflammation contribute to neuronal injury after subarachnoid hemorrhage (SAH). A disintegrin and metalloprotease with thrombospondin type 1 motif member 13 (ADAMTS13) cleaves von Willebrand factor multimers, and inhibits thrombus formation and seemingly inflammatory reactions.

Objective: We investigated the effect of ADAMTS13 in experimental SAH.

Methods: A total of 100 male C57/BL6 mice were randomly assigned to 4 groups: Sham (n=15), SAH (n=27), vehicle (n=25) and ADAMTS13 (n=23; 100µl per 10g body weight of 100µg ADAMTS13 per 1ml 0.9% NaCl; 20 min after SAH). Neurological performance was assessed at day 1 and 2 after SAH. Animals were sacrificed at day 2. The amounts of subarachnoid blood, microthrombi, apoptosis and degenerative neurons were compared. The degree of neuronal inflammation and vasospasm was also compared. In 5 mice each (SAH and ADAMTS13 group), bleeding time was assessed 2h after SAH.

Results: Systemic administration of ADAMTS13 achieved a significant amelioration of microthrombosis and improvement of neurological performance. ADAMTS13 reduced the amount of apoptotic and degenerative neurons. A tendency towards decreased neuronal inflammation was shown. ADAMTS13 did not show any significant effect on vasospasm. The degree of systemic inflammation was not changed by ADAMTS13 administration. ADAMTS13 neither increased the amount of subarachnoid blood nor prolonged the bleeding time.

Conclusions: ADAMTS13 may reduce neuronal injury after SAH by reducing microthrombosis formation and neuronal inflammation, thereby providing a new option to mitigate the severity of neuronal injury after SAH.

Key words: ADAMTS13, cerebral vasospasm, microcirculation, subarachnoid hemorrhage, thrombosis

Introduction

A subarachnoid hemorrhage (SAH), due to a ruptured intracranial aneurysm, is a common and frequently devastating condition, accounting 5-10% of all strokes with an incidence of 7-20 out of 100,000. Despite considerable advances in diagnosis and surgical and/or interventional treatment, outcome for patients with SAH remains poor. How to best deal with neuronal injury after aneurysmal SAH remains a challenging task in contemporary neurosurgery and neurocritical care. In the past decades, cerebral (macro-)vasospasm (CVS) has been regarded as the major cause of neurological deterioration and consequent poor outcome after SAH [1-3]. As neurological deterioration often occurs in a delayed fashion due to ischemia, the term 'delayed cerebral ischemia' (DCI) is frequently used. More recent evidence suggests that CVS is not the only cause of DCI and that the entire process of DCI may be multifactorial [1-4]. The following mechanisms have been recently proposed to contribute to the neuronal injury after SAH: 1) Activation of the coagulation cascade, in particular the von Willebrand Factor (vWF)-platelet axis; 2) occurrence of microthrombosis; and 3) reactive inflammation with cytokine release, such as Interleukin (IL)-6 and high-mobility group box (HMGB)1 [1, 2, 4-10]. A disintegrin and metalloprotease with thrombospondin type 1 motif member 13 (ADAMTS13) is a vWF cleaving protease that inhibits thrombus formation. ADAMTS13 has been described as a key protein in linking thrombosis with inflammation [11, 12]. Virtually no reports have been published about the role of ADAMTS13 after SAH, except for one clinical study in which reduced ADAMTS13 activity was reported in patients with DCI [13]. In experimental ischemic stroke models, ADAMTS13 deficiency resulted in an aggravation, and administration of ADAMTS13 in an amelioration of neuronal injury [14-17]. In the current study, we investigated whether ADAMTS13 administration would reduce microthrombosis and neuronal injury in mice after SAH.

Methods

Animals

Male C57/BL6 mice were used (22-26g body weight, Kyudo Co. Ltd., Japan). In the first of two separate experimental series, mice underwent experimental SAH and each 5 mice were sacrificed at day 1, 2 and 3 after SAH to establish a time course of microclot formation. Another 5 sham operated animals were sacrificed at day

2. Dead animals were replaced to achieve aforementioned numbers (n=5 each). In the second study, a total of 100, were used. The animals were handled according to the guidelines and regulations of the institutional animal care committee of Fukuoka University (Fukuoka, Japan). Experiments were performed in accordance with the good laboratory practice guidelines [18]. Mice were randomly assigned to 4 groups: Sham, SAH, vehicle and ADAMTS13.

Mouse SAH model

The circle of Willis perforation model was used [5, 6]. In brief, mice were anesthetized with isofluarane (Escain, Mylan Co. Ltd. Japan), 5% induction, 1.5% maintenance. The operating microscope had a 7- to 45-fold magnification (Arms Systems Co. Ltd., Japan). Standard microsurgical instruments including a bipolar forceps were used. In the prone position, a fiber-optic micro pressure transducer (Samba Sensors AB, Goteborg, Sweden) was placed in the left temporo-basis. In the supine position, a 5-0 monofilament nylon suture (Ethilon, Eticon, USA) was introduced through the external carotid artery and advanced towards the internal carotid artery. The filament was quickly withdrawn after perforation of the circle of Willis. In sham animals, the filament was advanced only until the tip was located intracranially to avoid vessel rupture. Body temperature was kept at 37.5°C, using a feedback-controlled heating pad (NS-TC10, Neuroscience Inc., Japan).

Drug Administration

In animals of the ADAMTS13 group, 100µl per 10g body weight of 100µg recombinant human (rh)ADAMTS13 (6156-AD, R&D Systems, USA) per 1ml 0.9% NaCl was administered 20min after SAH through the tail vein. In animals of the vehicle group, 100µl per 10g body weight of 0.9% NaCl was administered. The currently applied dose was derived from a previous study in experimental ischemic stroke, where multiple dosages were examined (M. Fujioka and K. Mishima, unpublished data).

Neurological Assessments

Neurological performance was assessed in a blinded fashion by the modified Garcia's score and tape removal test at day 1 and day 2 after SAH in the second experimental series. In brief, the Garcia's score examines spontaneous activity, motor function and reflexes [19]. For the tape removal test, standardized 5x5mm pieces of tape were placed on the plantar surface of the forepaw. The animals were given a maximum of 180sec per tape and side. This adhesive removal test is effective in detecting functional deficits after focal ischemia [17, 20].

Bleeding Time

In 10 mice (n=5 each in SAH and ADAMTS13 group), bleeding time was assessed 2h after SAH in a blinded fashion. Under anesthesia, a 3mm segment of the tail was amputated and immersed in PBS. The time required for the stream of blood to stop was defined as the bleeding time [17].

Euthanasia and Sampling

In the second experimental series, 51 animals (sham n=10, SAH n=13, vehicle n=13, ADAMTS13 n=15) were euthanized under deep anesthesia at day 2 after SAH by transcardial perfusion fixation (10ml ice cold 0.9% NaCl followed by 50ml of 4% paraformaldehyde). Day 2 was chosen as the time point at which the peak occurrence of microthrombosis was observed in the first experiment (Figure 1A), in concordance with a previous study [6]. The brains were removed, photographed and post-fixed. Severity of the bleeding was assessed using a grading system as previously described [21]. Automated degreasing, dehydration (RH-12, Sakura Seiko Co. Ltd., Japan) and paraffin embedding followed. Transcardial blood sampling was performed prior to euthanasia. Blood samples were centrifuged with 1,500g for 15min at 4°C, and the supernatant was stored at -30°C. In 25 animals (sham n=5, SAH n=8, vehicle n=6, ADAMTS13 n=6), the brains were removed without fixation and homogenized in a protein extraction solution (Pro-Prep, iNtRON Biotechnology, South Korea). The samples were centrifuged with 20,000g for 45min at 4°C, and the supernatant was stored at -80°C.

Histological Examinations

Embedded blocks were sliced into 6μm sections and mounted on glass slides. Hematoxylin and eosin (HE) staining was performed for CVS identification. The distal ICA was photographed at a 200x magnification under a light microscope. The lumen diameter and vessel wall thickness were quantified at four different points along the artery circumference and averaged using Image J software (Ver. 1.45s, NIH, USA) in a blinded fashion.

Microthrombi were visualized by immunohistochemistry (IHC) using fibrinogen staining: After heat-induced antigen retrieval, the slides were incubated in sheep anti-fibrinogen antibody (1:200) (LifeSpan BioSciences, USA) for 2h. Then incubation in biotinylated secondary anti-sheep antibody (1:500) followed for 1h. Staining was visualized with VECTASTAIN Kit (Vector, USA) and counterstained with hematoxylin. Predefined regions of interest (ROI)s -3 hippocampal and 5 cortical regions in each hemisphere- were photographed with a 200x magnification. The cumulative number of microthrombi -regardless of size- was counted in a blinded fashion.

HMGB1 protein and neuronal nuclei (NeuN) double staining was performed to qualitatively evaluate the degree of neuronal inflammation [15, 22]: After antigen retrieval, the slides were incubated in rabbit anti-HMGB1 (Abcam, USA) and biotinylated mouse anti-NeuN antibody (Chemicon, USA) overnight. An incubation with DyLight 488 conjugated donkey anti-rabbit (Abcam, USA) and Texas Red conjugated avidin (NeutrAvidin, Invitrogen, USA) followed for 1h. The ROIs were photographed under a fluorescence microscope and assessed.

For the qualitative assessment of neuronal injury after SAH, Fluoro-Jade C (FJC) staining (Merck Millipore, USA) with diamino-phenylinodole (DAPI) (Invitrogen, USA) counterstaining was performed, as described before [23]. To evaluate the occurrence of apoptotic neurons, terminal deoxynucleotidyl transferase dUTP nick labeling (TUNEL) staining was performed (Roche Diagnostics, Germany). Staining was performed according to the manufacturers' instruction manual. The amounts of FJC and TUNEL positive cells were qualitatively evaluated in ROIs in a blinded fashion.

Enzyme-linked Immunosorbent Assay

IL-6 (Quantikine mouse IL-6 immunoassay, R&D Systems, USA), HMGB1 (HMGB1 ELISA kit II, Shino-Test Corporation, Japan) and ADAMTS13 (Quantikine human ADAMTS13 immunoassay, R&D Systems, USA) were quantitatively measured in blood and homogenized brain supernatant. Total protein in supernatant was calculated by the Bradford protein assay and cytokine levels were calculated per mg of total protein. All concentrations below the minimal detection limit were assigned to the detection limit.

Statistical Analysis

Nominal variables were given as frequency and percentage, and continuous variables by mean ±SEM. Nominal variables were compared by the Chi squared test. Ordinal variables (Garcia's score) were compared by a non-parametric ANOVA. Continuous variables were compared by ANOVA, with logarithmic transformation in case of need. For comparison within the group, ANOVA for repeated measures was applied (first experimental series). A p-value <0.05 was regarded as statistically significant. IBM SPSS Statistics 20.0 software was used.

Results

Mortality, Bleeding Time and Degree of Hemorrhage

In the first experimental series, the mortality was n=3 (20%) within 72h. In the second experimental series, a total of 10 animals were excluded (intrasurgical fatality before induction of SAH, n=3; no or questionable induction of SAH n=4; failed drug administration n=1; accidental ICA occlusion n=1; failed tissue sampling n=1) leaving sham n=15; SAH n=27; vehicle n=25; ADAMTS13 n=23 for analysis. The vessel perforation resulted in a sharp increase of ICP, confirming a successful SAH (Figure 1B and C). At day 2 after SAH, a tendency towards lower mortality was found in the ADAMTS13 group. Mortality was n=0 (0%) in the sham, n=6 (22%) in SAH, n=6 (24%) in vehicle, and n=3 (9%) in the ADAMTS13 group (p=0.12). The bleeding time between SAH and ADAMTS13 group did not differ: 103.2 ±20.8 vs. 100.8 ±21.4sec (p=0.94). The degree of hemorrhage was not different between animals in the SAH, vehicle and ADAMTS13 groups (p=0.42).

Neurological Performance

Animals in the SAH and vehicle groups did neurologically worse compared to the sham group as assessed by Garcia's score and tape removal test (both p<0.01). Administration of ADAMTS13 significantly improved the neurological performance (p<0.05). Results are shown in Figure 2.

Time Course of microthrombus formation

The first experimental study showed that the cumulative numbers of fibrinogen-positive microthrombi were significantly higher on day 2 and 3, but not day 1, in animals subjected to SAH compared to sham animals. Within the SAH group, the cumulative number of microthrombi was higher on day 2, compared to day 1 and 3, in concordance with a previously published study [6]. The results are shown in Figure 1A.

Effect of ADAMTS13 on the Occurrence of Microthrombosis and CVS

Immunohistochemical examinations showed the presence of microthrombi in both the cortical and hippocampal regions at day 2 after SAH (Figure 3A). The cumulative amount of microthrombi was significantly higher in the SAH, vehicle, and ADAMTS13 groups as compared to the sham group (p<0.05). Administration of ADAMTS13 significantly reduced the amount of microthrombi as well in the cortex as in the hippocampal region, compared to SAH and vehicle (p<0.01). However, the amount remained significantly higher compared to

sham animals in the cortex ($p<0.05$). The results are shown in Figure 3B. Signs of CVS could be seen in animals subjected to SAH (Figure 3C). The lumen diameter/wall thickness ratio was significantly higher in sham animals compared to SAH, vehicle and ADAMTS13 ($p<0.05$). Administration of ADAMTS13 did not resolve the morphological signs of CVS (Figure 3C and D).

Effect of ADAMTS13 on Inflammation

Immunohistochemical examination showed a translocation of HMGB1 from the neuronal nuclei to cytoplasm in animals of the SAH and vehicle groups. In ADAMTS13 treated animals, translocation was less often observed, indicating an amelioration of neuronal inflammation (Figure 4). Animals developing hemorrhage showed higher cerebral HMGB1 levels, compared to sham animals. In the ADAMTS13 group, the increase in cerebral HMGB1 was less pronounced. However, only a statistical tendency could be found ($p=0.10$). Cerebral IL-6 levels showed a similar pattern, but without statistical significance ($p=0.29$). Systemic IL-6 levels were significantly increased in animals subjected to SAH ($p<0.05$). However, systemic IL-6 levels in animals subjected to SAH did not differ. Systemic HMGB levels were higher in the SAH and vehicle groups compared to the sham and ADAMTS13 ones, but without significance ($p=0.27$). Systemic levels of ADAMTS13 were under the mean minimal detection limit in the sham, SAH and vehicle animals, while the presence of rhADAMTS13 could be verified in the ADAMTS13 group. No ADAMTS13 was detected in brain supernatants. The results are shown in Table 1.

Effect of ADAMTS13 on Neuronal Injury

In animals of the SAH and vehicle groups, neuronal cell death and degeneration were detected to the same extent in the parietal and temporal cortex as in the hippocampal regions by FJC staining. Administration of ADAMTS13 resulted in a decreased number of FJC positive cells (Figure 5). TUNEL positive cells, which appeared to be neurons, were found in the outer 2 layers of the temporal and parietal cortex of SAH and vehicle animals. ADAMTS13 administration reduced the amount of apoptotic cells (Figure 5).

Discussion

Neuronal injury after SAH has been associated with the occurrence of CVS, which is a morphological narrowing of large cerebral arteries [2]. However, newer clinical evidence suggests that treatment of CVS does not necessarily prevent the occurrence of DCI with consequent poor outcome [2, 4]. The dissociation between CVS and outcome became evident in well-designed clinical trials using the endothelin-1 receptor antagonist clazosentan [2, 3]. Current evidence suggests that the mechanisms underlying neuronal injury are multifactorial [1-3]. Activation of the coagulation cascade with occurrence of microthrombi and inflammatory response are frequently discussed potential protagonists [4-8, 13, 24]. Accumulating evidence suggests that a complex series of cellular and molecular events are elicited by the presence and breakdown of erythrocytes in the subarachnoid space, leading to an inflammatory response [4, 8, 10, 24]. In the acute phase of inflammation, adhesion molecules are expressed, leading to leukocyte adherence at the endothelium with subsequent activation and migration. Cytokines are powerful mediators and regulators of inflammation. Cytokines stimulate the expression of adhesion molecules and further propagate inflammation. Proinflammatory cytokines, such as IL-6, induce coagulation and might contribute to microthrombosis formation [4, 11]. Several adhesion molecules and cytokines have been associated with CVS, DCI and/or poor outcome, IL-6 and HMGB1 among others [8-10, 24]. Interestingly, clazosentan alleviated CVS but did not affect the occurrence of microthrombi or amount of neuronal injury ad day 2 after experimental SAH [6, 25].

In the current study, important pathologies could be reproduced. These include CVS, microthrombosis, neuronal injury and neurological deficits. Administration of rhADAMTS13 after SAH produced a significant amelioration of microthrombosis and improvement of neurological performance. The amount of apoptotic and degenerative neurons was decreased. A tendency towards decreased neuronal inflammation was noted. ADAMTS13 administration did not exert any significant effect on CVS of the large arteries, and the degree of systemic inflammation was not significantly affected. Increased rhADAMTS13 levels after 48h confirmed its successful administration in view of its reported long circulatory half-life [26]. ADAMTS13 prevents microvascular thrombosis by cleaving ultra-large (UL)vWF multimers, which are extremely thrombogenic, into smaller less active multimers. Deficiency of ADAMTS13 causes thrombotic thrombocytopenic purpura (TTP), which is associated with the occurrence of microthrombosis followed by ischemic complications, such as cerebral infarction [27]. The vWF-ADAMTS13 axis modulates inflammation and has recently been described as a “new link between thrombosis and inflammation” [12]. ADAMTS13 deficiency increases vWF-dependent

leukocyte rolling, adhesion and extravasation in experimental inflammation [12, 16]. In experimental ischemic stroke, a couple of studies showed that ADAMTS13 plays a major role in determining the extent of cerebral injury: ADAMTS13 deficiency resulted in neurological worsening [14, 15, 17], enlarged infarct size [14-17], increased leukocyte infiltration [14, 16, 17], and increased expression of proinflammatory cytokines, such as IL-6 and HMGB1 [15-17]. Importantly, infusion of rhADAMTS13 improved neurological performance, reduced infarct size and neither prolonged the bleeding time nor promoted brain hemorrhage [17]. Further experiments using ADAMTS13 deficient mice with prior immunodepletion of neutrophils and vWF deficient mice demonstrated a causal role for inflammation in the enhanced brain injury that occurred in the presence of ADAMTS13 deficiency [16]. As mentioned above, virtually no reports are available about the role of ADAMTS13 in neuronal injury after SAH, except for one clinical observational study. In the latter, patients with DCI had a more marked decrease in ADAMTS13 activity, and a more profound increase in vWF activity. The results suggest that microthrombosis plays a role in the pathogenesis of neuronal injury after SAH, as a result of decreased ADAMTS13 activity [13]. However the mechanism by which ADAMTS13-activity decreases remains unclear. The author discussed, that the activity might be suppressed by IL-6, because IL-6 inhibits the cleavage of ultra-large vWF by ADAMTS13 [11].

The results of the current study provide further evidence supporting the involvement of ADAMTS13 in the pathogenesis of microthrombosis and neuronal injury after SAH. Taking the current knowledge -including the present results- into account, the following mechanism might be proposed: An SAH event triggers both a systemic and compartmental (within the central nervous system) inflammatory response with cytokine release. This inflammatory response activates the coagulation cascade and decreases ADAMTS13 activity. As a consequence, platelet thrombi form, and without negative feedback regulation by proteolysis of vWF by ADAMTS13, progressive thrombus growth may narrow and ultimately plug microvascular lumina. The platelet-vWF string directly supports leukocyte transmigration, which results in further amplification and propagation of inflammation, initiating a vicious circle.

A meta-analysis of randomized trials showed a trend towards better outcome in patients treated with antiplatelet agents, possibly due to a reduction in the development of DCI. However, the results were not statistically significant, with the authors noting that the hemorrhagic complications of these drugs possibly counterbalancing the beneficial effects [28]. Treatment with antiplatelet agents to prevent DCI or poor outcome is currently not recommended, and more-specific strategies are required. In this context ADAMTS13 might be of interest, as it might not lead to a higher risk of bleeding.

The current study has several limitations. The results do not prove a causal relationship between microthrombosis and neuronal injury, although it might be reasonable to assume one, as a quantitative correlation between the numbers of microthrombi and apoptotic neurons has been described [7]. Further, the effect of ADAMTS13 administration on the inflammatory response after SAH remains difficult to interpret. The IL-6 and HMGB1 levels within the brain showed only a statistical tendency, indicating an amelioration of neuroinflammation. This might be explained as follows: 1.) cytokine concentrations were measured in the parenchyma and not in the subarachnoid space, i.e. cerebrospinal fluid (CSF) compartment, where the inflammatory response is suggested to be the strongest. Accordingly, IL-6 levels in the parenchyma were less elevated than in the CSF in patients with SAH [10]. 2.) ADAMTS13 leads to an amelioration of reactive inflammation, but might not suppress the initial inflammatory response caused by the hemorrhage. Questions remain as to whether the intimated decrease of neuroinflammation is a direct effect of ADAMTS13 and whether inflammation leads directly to neuronal injury. Subsequent studies using ADAMTS13 deficient mice with and without immunodepletion may provide additional information.

In conclusion the current study showed that systemic application of ADAMTS13 resulted in a significant amelioration of microthrombosis and improvement of neurological performance. Administration of ADAMTS13 reduced the amount of apoptotic and degenerative neurons. A tendency towards decreased neuronal inflammation was shown. ADAMTS13 might have a relatively low risk of hemorrhagic complications. Therefore, we propose that ADAMTS13 may offer a new option for the prophylaxis of cerebral injury after SAH.

Authorship

Contribution: C. Muroi designed the study, performed most of the experiments, analyzed the results, and prepared the manuscript. K. Mishima, K. Okuchi, Y. Fujimura, J. Fandino, E. Keller, K. Iwasaki and M. Fujiwara provided direction throughout the work, made the overall experimental design, and edited the manuscript. M. Fujioka and T. Nakano helped to perform the animal experiments, in particular the neuronal assessments. M. Fujioka, K. Mishima and K. Irie provided direction in histological staining procedures and ELISA measurements.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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Figure Legends

Fig. 1. (A) Cumulative amount of microthrombi shown as bar graphs. The cumulative numbers of fibrinogen-positive microthrombi were significantly higher on day 2 and 3, but not day 1, in animals subjected to SAH compared to sham animals. Within the SAH group, the cumulative number of microthrombi was higher on day 2, compared to day 1 and 3. (B) Animals subjected to SAH showed a sharp increase of ICP. (C) Representative brain specimen showing successful SAH with hematoma in the basal cisterns (upper picture). No hemorrhage was present in sham animals (lower picture).

Fig. 2. Neurological assessment by Garcia's score (A) and tape removal test (B) shown as bar graphs. All 3 groups subjected to SAH did neurologically worse compared to sham ($p<0.01$ and $p<0.05$). Administration of ADAMTS13 improved neurological performance compared to animals in the SAH and vehicle groups (both $p<0.01$). However, performance remained significantly worse compared to sham animals (both $p<0.01$).

Fig. 3. (A) Representative images from histochemical visualization of microthrombi (arrows). Scale bar: 100μm. (B) Number of microthrombi shown as bar graphs. Administration of ADAMTS13 significantly decreased the amount of microthrombi (arrow). (C) Representative images of the distal ICA showing morphological signs of CVS in animals of the vehicle and ADAMTS13 groups. Note the presence of subarachnoid blood (arrows). Scale bar: 50μm. (D) Bar graphs showing significant higher lumen diameter/wall thickness ratio in all 3 groups subjected to SAH compared to sham.

Fig. 4. Representative images from HMGB1 (green)/NeuN (red) double staining of the ipsilateral cortex. In sham animals, HMGB1 remained predominantly nuclear (left column). Induction of SAH induced HMGB1 translocation from the nucleus (middle column, arrow, higher magnification). Translocation of HMGB1 was less observed in animals of the ADAMTS13 group (right column). Scale bar: 100μm.

Fig. 5. Representative images of FJC (green)/DAPI (blue) double staining (upper 2 rows) and TUNEL staining (bottom row). Degenerative neurons were present after SAH as well in the hippocampus as in the cortex (middle column, upper 2 rows, arrows). TUNEL positive neurons (brown) were present after SAH in the cortex (middle column, bottom row, arrows). Less FJC and TUNEL positive neurons were observed in animals of the ADAMTS13 group (right column, arrows). Scale bar: 100μm.

Table 1. Inflammatory parameter and ADAMTS13 levels

	Sham	SAH	Vehicle	ADAMTS13	Sig.
Cerebral HMGB1 [ng/mg]	637.5 ±150.1	1069.7 ±128.6	1030.7 ±168.8	822.4 ±134.9	p=0.10
Cerebral IL-6 [fg/mg]	255.5 ±86.6	525.8 ±145.2	576.6 ±200.4	393.0 ±154.3	p=0.29
Systemic HMGB1 ng/ml	6.3 ±0.5	9.5 ±2.0	8.5 ±2.6	5.5 ±0.5	p=0.27
Systemic IL-6 pg/ml	6.3 ±0.9	40.8 ±9.4	36.2 ±7.4	33.2 ±9.7	p<0.05
Systemic ADAMTS13 ng/ml	0.78 ±0.0	0.78 ±0.0	0.78 ±0.0	46.0 ±20.1	p<0.01

Sig. indicates significance.

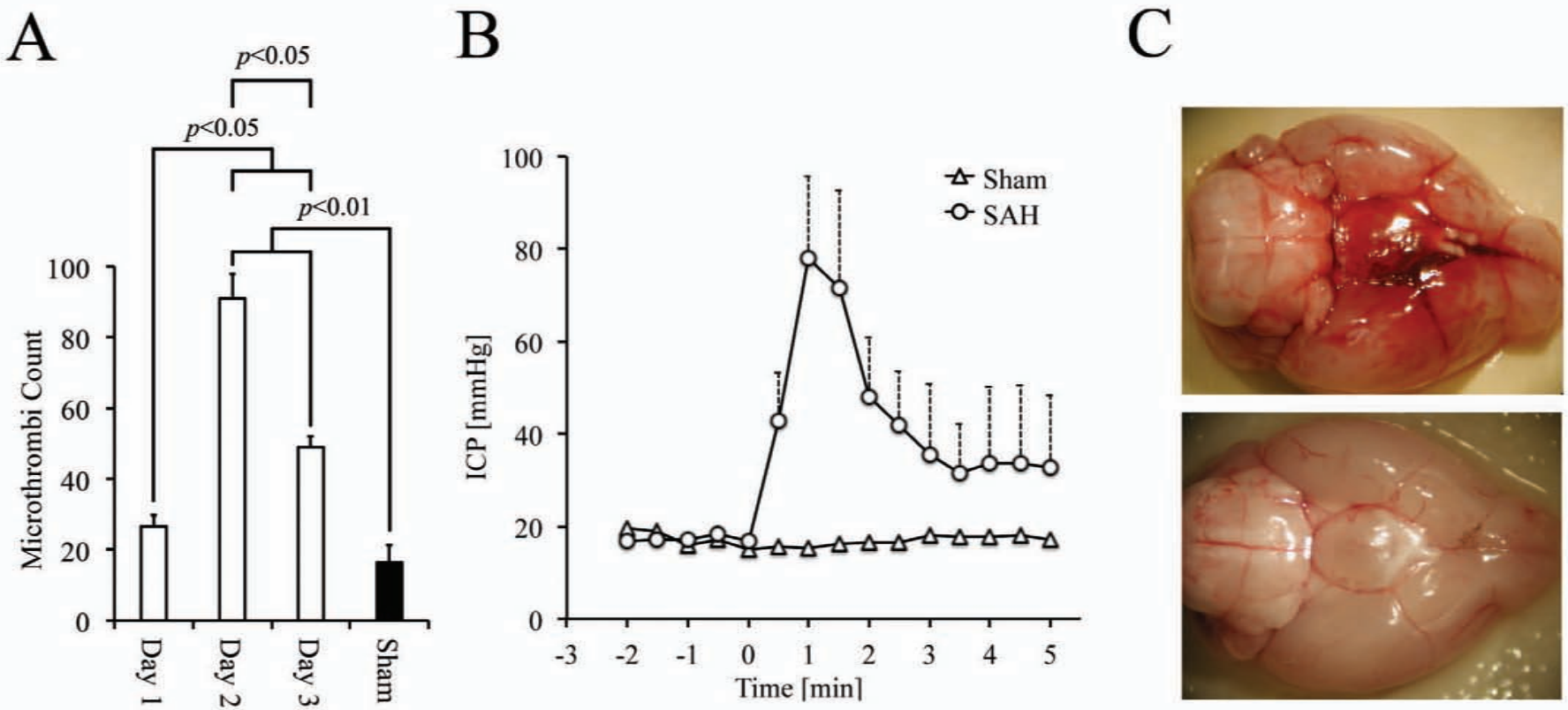
A
p<0.05
p<0.05
p<0.01
Microthrombi Count
100
80
60
40
20
0
Day 1
Day 2
Day 3
Sham
B
ICP [mmHg]
100
80
60
40
20
0
Sham
SAH
-3
-2
-1
0
1
2
3
4
5
Time [min]
C

Figure 1

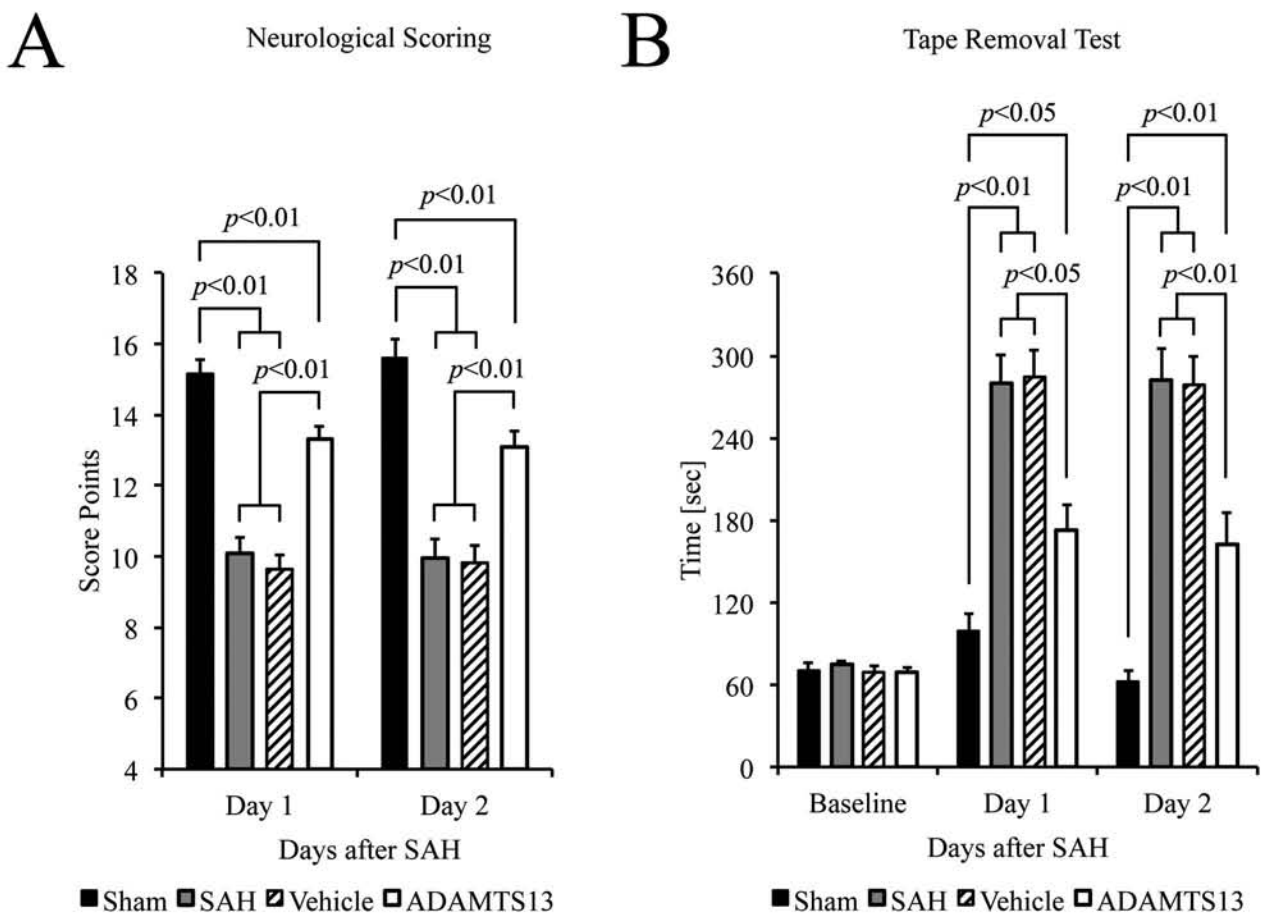
A
Neurological Scoring
Score Points
18
16
14
12
10
8
6
4
p<0.01
p<0.01
p<0.01
p<0.01
p<0.01
p<0.01
Day 1
Day 2
Days after SAH
Sham
SAH
Vehicle
ADAMTS13
B
Tape Removal Test
Time [sec]
360
300
240
180
120
60
0
p<0.05
p<0.01
p<0.01
p<0.05
p<0.01
Baseline
Day 1
Day 2
Days after SAH
Sham
SAH
Vehicle
ADAMTS13

Figure 2

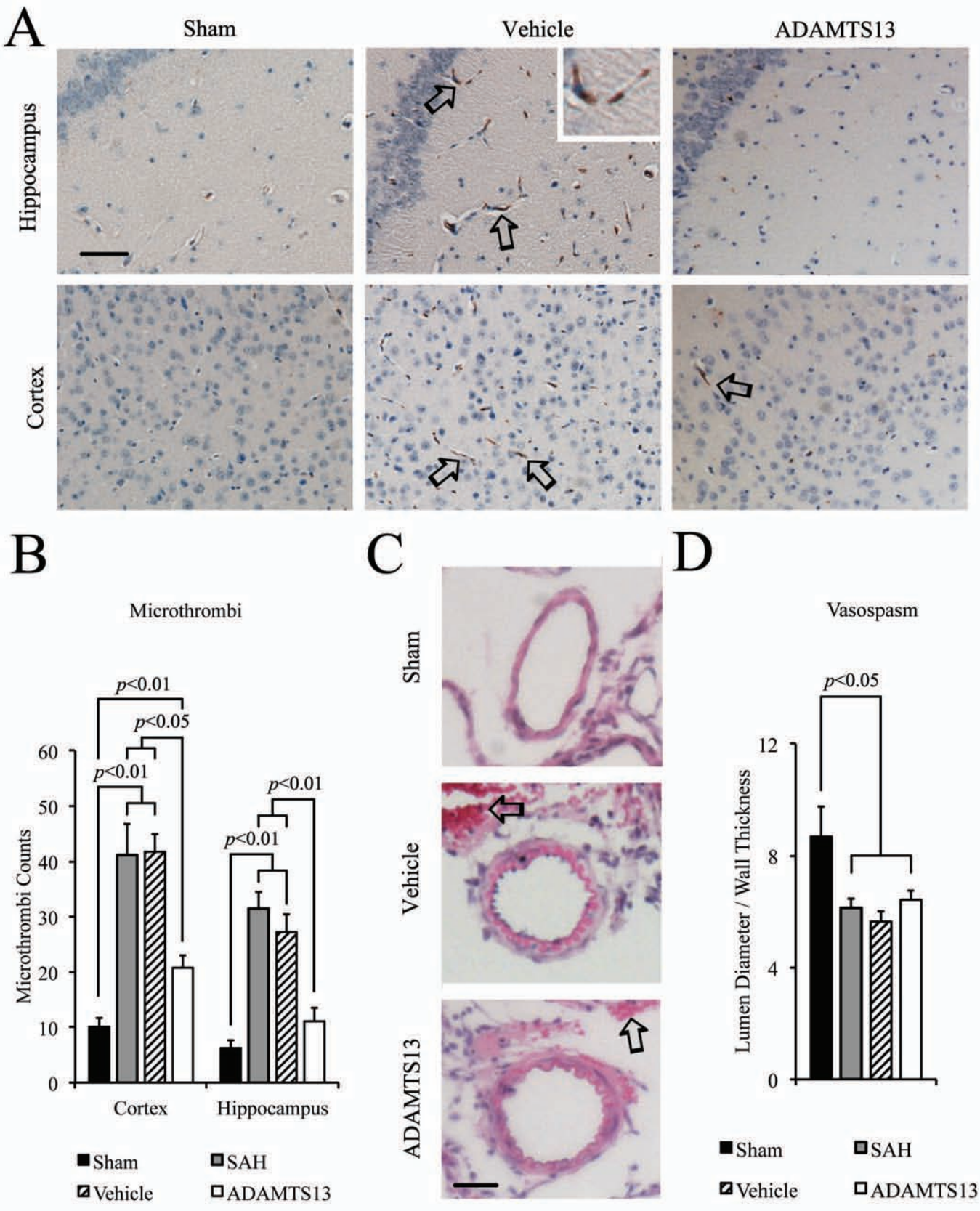
A
Sham
Vehicle
ADAMTS13
Hippocampus
Cortex
B
Microthrombi
p<0.01
p<0.05
p<0.01
p<0.01
p<0.01
Microthrombi Counts
60
50
40
30
20
10
0
Cortex
Hippocampus
Sham
SAH
Vehicle
ADAMTS13
C
Sham
Vehicle
ADAMTS13
D
Vasospasm
p<0.05
Lumen Diameter / Wall Thickness
12
8
4
0
Sham
SAH
Vehicle
ADAMTS13

Figure 3

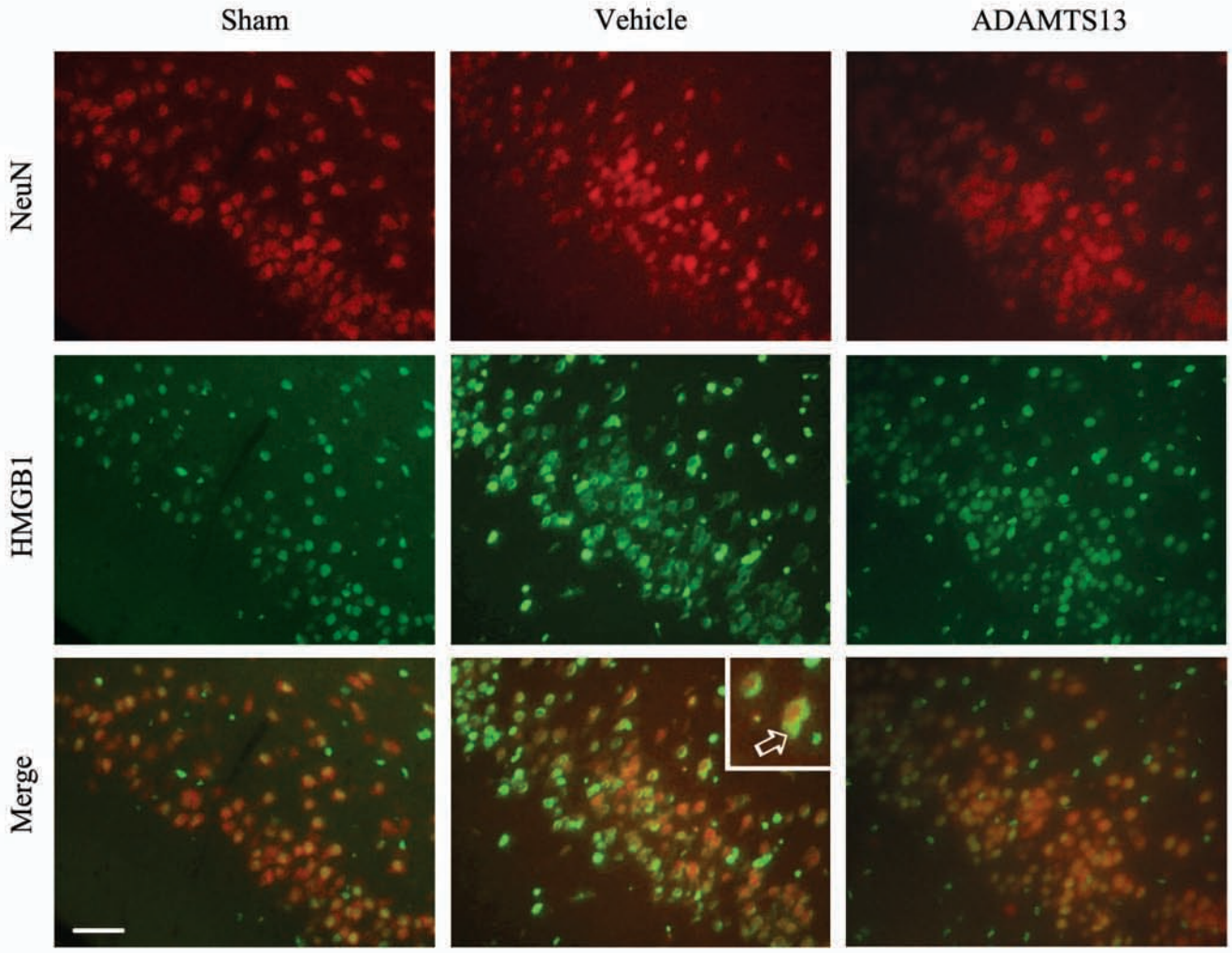

Figure 4

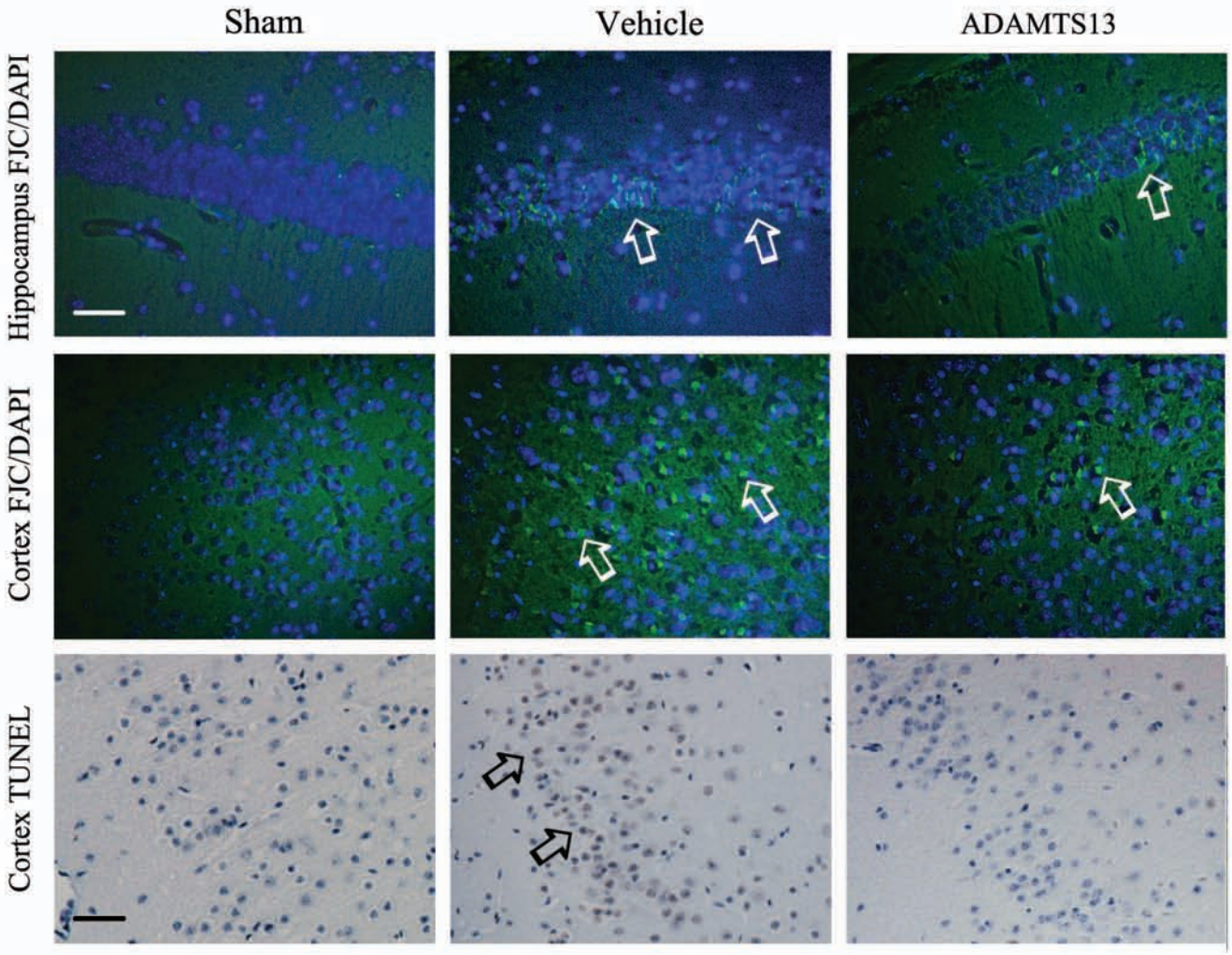
Sham
Vehicle
ADAMTS13
Hippocampus FJC/DAPI
Cortex FJC/DAPI
Cortex TUNEL

Figure 5